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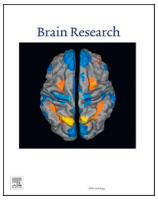

Modulation of neuronal morphology by antipsychotic drug: Involvement of serotonin receptor 7

Luisa Speranza [a,1], Marta Molinari [b,1], Floriana Volpicelli [a,*], Enza Lacivita [c], Marcello Leopoldo [c], Salvatore Pulcrano [d], Gian Carlo Bellenchi [d], Carla Perrone Capano [a,1], Marianna Crispino [e,1]

[a] Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, 80131 Naples, Italy
[b] Telethon Institute of Genetics & Medicine, Via Campi Flegrei, 34, 80078 Pozzuoli, Naples, Italy
[c] Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari Aldo Moro, 70125 Bari, Italy
[d] Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, 80131 Naples, Italy
[e] Department of Biology, University of Naples Federico II, 80126 Naples, Italy

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ABSTRACT

Antipsychotic drugs (APDs) are the primary pharmacological treatment for schizophrenia, a complex disorder characterized by altered neuronal connectivity. Atypical or second-generation antipsychotics, such as Risperidone (RSP) and Clozapine (CZP) predominantly block dopaminergic D_2 and serotonin receptor 2A (5-HT2A) neurotransmission. Both compounds also exhibit affinity for the 5-HT7R, with RSP acting as an antagonist and CZP as an inverse agonist. Our study aimed to determine whether RSP and CZP can influence neuronal morphology through a 5-HT7R-mediated mechanism. Here, we demonstrated that CZP promotes neurite outgrowth of early postnatal cortical neurons, and the 5-HT7R mediates its effect. Conversely, RSP leads to a reduction of neurite length of early postnatal cortical neurons, in a 5-HT7R-independent way.

Furthermore, we found that the effects of CZP, mediated by 5-HT7R activation, require the participation of ERK and Cdk5 kinase pathways. At the same time, the modulation of neurite length by RSP does not involve these pathways.

In conclusion, our findings provide valuable insights into the morphological changes induced by these two APDs in neurons and elucidate some of the associated molecular pathways. Investigating the 5-HT7R-dependent signaling pathways underlying the neuronal morphogenic effects of APDs may contribute to the identification of novel targets for the treatment of schizophrenia.

1. Introduction

Antipsychotic drugs (APDs) are psychotropic medications mainly used to treat psychosis and, in particular, schizophrenia (Mackin and Thomas, 2011). Schizophrenia is a multifactorial disabling mental illness affecting about 1 % of the population, characterized by a wide array of anomalies in the structure of the nervous system, as well as alterations in neurotransmission and connectivity (Lewis and Lieberman, 2000; Stachowiak et al., 2013). The etiology and pathogenesis of schizophrenia are intricate and mostly unknown, involving a complex interplay between genetic predispositions and environmental factors that interfere with the brain's development (Lewis and Sweet, 2009). Schizophrenia is typically diagnosed in early adulthood, but numerous studies indicate that the alterations begin early in neurodevelopment, suggesting that this disorder can be considered a neurodevelopmental disorder (Murray and Lewis, 1988; Owen et al., 2011). Indeed, schizophrenia shares many risk genes with neurodevelopmental disorders, such as autism spectrum disorders, intellectual disability, and attention-deficit hyperactivity disorders (Owen et al., 2011; Rees and Kirov, 2021). Furthermore, genes associated with autism and schizophrenia regulate dendritic morphogenesis, and recent research in the pharmacological field is making significant progress in improving neuronal cytoarchitecture deficits (Copf, 2016).

Although APDs are the leading treatment for schizophrenia, they

* Corresponding author at: Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, 80131 Naples, Italy.
E-mail address: floriana.volpicelli@unina.it (F. Volpicelli).
[1] These authors contributed equally to this work.

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display variable clinical effectiveness, tolerability, and side effects of different severity. APDs are commonly classified into three groups: typical or first-generation (e.g., chlorpromazine, trifluoperazine, and haloperidol), atypical or second-generation (e.g., clozapine, olanzapine, and risperidone), and third generation (e.g., aripiprazole and cariprazine). Despite this simple scheme, the differences among the three groups are difficult to delineate. First-generation antipsychotics have been used since the 1950 s, and primarily act as dopamine D_2 receptor antagonists. They are mainly associated with extrapyramidal side effects (EPS), such as tardive dyskinesia, dystonia, and parkinsonism (Mackin and Thomas, 2011). Atypical or second-generation antipsychotics, introduced in the 1990 s, have a broader range of action and a multi-receptor profile. They mainly act as serotonin receptor 2A (5-HT2A) and dopamine D_2 antagonists (Chokhawala and Stevens, 2023). They also affect the glutamatergic system by reducing glutamatergic hyperactivity through serotonergic control of glutamate release in the cortex (Stefani and Moghaddam, 2005). Second-generation antipsychotics feature less severe EPS than first-generation antipsychotics but show some metabolic side effects (Gründer et al., 2009; Mackin and Thomas, 2011).

Clozapine (CZP) was the first drug to be discovered as an atypical antipsychotic, and it has been proven to be more effective than other antipsychotics in treating negative, cognitive, and depressive symptoms of schizophrenia (Fernández-Miranda et al., 2022; Mailman and Murthy, 2010). Clozapine undergoes extensive hepatic metabolism, forming, through the enzymatic action of cytochrome CYP1A2, norclozapine (desmethylclozapine) as the primary metabolite. Norclozapine retains some pharmacological activity, but it is generally less potent than clozapine itself. CZP is effective on negative symptoms of schizophrenic patients, improving memory, verbal learning, verbal fluency, and psychomotor speed. Serious side effects of CZP are the risk of agranulocytosis, seizures, myocarditis, other adverse cardiovascular and respiratory effects, and increased mortality in elderly patients with dementia-related psychosis. Due to this severe side effect profile, CZP is typically used in cases where other antipsychotic interventions have proven ineffective (Prior and Baker, 2003). CZP has an extremely complex polypharmacological profile with significant activity and high affinity for the 5-HT2A and the dopamine receptors D_1, D_2, and D_4. It was observed that CZP or other second-generation antipsychotics block the 5-HT2A receptor with higher potency than the D_2 receptor. Additionally, CZP affects some other serotonergic receptors, such as 5-HT1A and 5-HT7R, as well as histaminergic H_1, muscarinic M_1, α-adrenergic receptors (Wenthur and Lindsley, 2013). Significantly, in vivo CZP - acting as an agonist on 5-HT1A - increases dopamine release in the prefrontal cortex (PFC) (Bortolozzi et al., 2010; Díaz-Mataix et al., 2005; Ichikawa et al., 2001; Rollema et al., 1997). Moreover, it has been demonstrated that CZP, repeatedly administered at a lower dose, increases the D_2-5-HT1A and 5-HT1A–5-HT2A heterodimerization in the mouse PFC and FC (Szlachta et al., 2018).

Risperidone (RSP), a benzisoxazole derivative developed in 1993, is another atypical antipsychotic structurally and pharmacologically different from CZP (Carli et al., 2021). RSP exerts its pharmacological effects by binding to high-affinity serotonin and dopamine receptors and inhibiting them. It displays a high affinity for 5-HT2A, dopaminergic D_2, and α_1 and α_2 adrenergic receptors, low/medium affinity for other serotonergic receptors (5-HT2CR, 5-HT1AR, 5-HT1CR, 5-HT1DR, and 5-HT7R), and a low affinity for histaminergic H_1 receptors (Asenjo-Lobos et al., 2018). RSP is metabolized by the liver primarily by CYP2D6, an enzyme belonging to the cytochrome P450 superfamily of enzymes responsible for the metabolism of lipids, hormones, toxins, and drugs. The primary active metabolite of RSP is 9-hydroxyrisperidone, also known as paliperidone, which undergoes further metabolism, primarily via glucuronidation. Both risperidone and paliperidone contribute to the overall pharmacological effects observed during RSP treatment (Puangpetch et al., 2016).

In general, CZP and RSP are commonly used in the treatment of schizophrenia and related disorders, primarily by blocking dopaminergic and serotonergic signaling pathways. Nonetheless, their action mechanism is still partially unknown, thus it is of great relevance to investigate the cellular and molecular pathways underlying their actions.

Previous studies have indicated that both drugs have affinity for the 5-HT7R: RSP irreversibly binds and antagonizes 5-HT7R (Smith et al., 2006), while CZP is an inverse agonist or non-inactivating antagonist of the 5-HT7R (Andressen et al., 2015; Krobert and Levy, 2002). The 5-HT7R is involved in various functions, of the nervous system including modulation of neurotransmitter release, regulation of mood and emotion, and learning and memory (Crispino et al., 2020; Speranza et al., 2015, 2013; Volpicelli et al., 2019). Consequently, the alteration of 5-HT7R and its signaling pathway is linked to numerous neuropathologies and (Crispino et al., 2020; Speranza et al., 2015, 2013; Volpicelli et al., 2019). In particular, it was demonstrated that serotonin dysregulation is implicated in schizophrenia's pathophysiology (Tsegay et al., 2020). In line with this, emerging evidence underscored the potential relevance of targeting the 5-HT7R to improve the efficacy of antipsychotic medications for schizophrenia (Modica et al., 2018; Nikiforuk, 2015). However, the precise mechanisms underlying this interaction remain elusive, especially due to the variable responses of different patients (Nikiforuk, 2015).

To this aim, here we investigate whether RSP and CZP can modulate neuronal cytoarchitecture in embryonic and postnatal neuronal cultures from the mouse cortex, a brain region particularly affected in schizophrenic patients, and whether these effects are 5-HT7R-mediated.

2. Materials and methods

2.1. Animals

Timed pregnant C57BL/6 mice were housed, cared for, and sacrificed following the recommendations of the European Commission (UE 2010/63/UE). All the procedures related to animal treatments were approved by the Ethic-Scientific Committee for Animal Experiments and the Italian Ministry of Health (authorization N.491/2017-PR). Every effort was made to minimize animal suffering and to reduce the number of animals used by following the principles of the 3Rs (Replacement, Reduction, Refinement). The animals were bred in-house at the Institute of Genetics and Biophysics "Adriano Buzzati Traverso," CNR, Naples, Italy.

2.2. Embryonic and postnatal neuronal primary cultures

The embryonic age (E) was determined by the date of insemination (i.e., the appearance of the vaginal plug was considered as day E0). About 15–20 embryos from different dams were pooled for every cell culture preparation. The prefrontal cortex (CTX) was dissected from E15 embryos under a stereoscope in sterile conditions, and placed in PBS without calcium and magnesium supplemented with 33 mM glucose. Dissociated cells were cultured as previously described (Di Porzio et al., 1980; Speranza et al., 2013; Volpicelli et al., 2004). Briefly, the dissected areas were enzymatically dissociated by incubation for 30 min at 37 °C in papain solution (Worthington, 20 U/ml, Milan, Italy) in Earle's balance salts containing 1 mM EDTA (Sigma-Aldrich, Milan, Italy), 1 mM cysteine (Sigma-Aldrich) and 0.01 % pancreatic DNAse (Sigma-Aldrich). After the addition of 1 mg/ml of bovine serum albumin (BSA, Sigma-Aldrich) and 1 mg/ml ovomucoid (OVM, Sigma-Aldrich), the cell suspensions were centrifuged 5 min at 800 rpm, resuspended in fresh plating medium and counted (Fiszman et al., 1991). For the viable cell count, the cell suspension was diluted 1:1 with 0.1 % trypan blue dye (Sigma-Aldrich).

For postnatal cell culture preparation, prefrontal cortices from newborn mice (P0-P3) were collected in HBSS (ThermoFisher Scientific, Milano, Italy) and enzymatically digested by incubation for 30 min at 37 °C in papain/DMEM solution (10 U/ml, Worthington Biochemical

Corporation, Lakewood, NJ 08701, USA) containing 50 mM EDTA, 100 mM $CaCl_2$, 2 mg/ml cysteine and 0.01 % pancreatic DNAse. The enzymatic digestion was blocked with 25 mg/ml albumin, and 25 mg/ml ovomucoid in 10 % FBS-DMEM medium added with 50 U/ml penicillin and 50 mg/ml streptomycin. The resuspended cells were mechanically dissociated in DMEM supplemented with 10 % FBS, and the viable cells were determined by trypan blue dye exclusion as described before.

Dissociated cells were plated at a 75 × $10^3/cm^2$ density for morphological analyses in Lab-Tek chamber slides (Nunc) coated with 15 μg/ml of poly-D-Lysine dissolved in water (Sigma-Aldrich).

Embryonic cells were grown in serum-free Neurobasal medium (ThermoFisher Scientific), supplemented with B27 (ThermoFisher Scientific), 2 mM L-glutamine (Sigma-Aldrich), penicillin (50 U/ml, Sigma-Aldrich) and streptomycin (50 μg/ml, Sigma-Aldrich).

Postnatal cells were grown in Neurobasal A-medium added with B27, 1 % FBS, 2 mM Glutamax, 50 U/ml penicillin, and 50 mg/ml streptomycin. The day after the medium was replaced with the same media, but without serum. Cells were maintained for 3 days in vitro (DIV) at 37 °C in a humidified incubator in the presence of 5 % CO_2, before experimental manipulation. For each experimental point, cultures were prepared at least in independent triplicates and were repeated using distinct culturing sessions.

2.3. Drug treatments

Neuronal cultures, after 3 days in vitro, were treated for 4 h with the following drugs: the 5-HT7R selective agonist LP-211 (100 nM, gifted by Prof. M. Leopoldo, University of Bari), the 5-HT7R selective antagonist SB-269970 (100 nM, Tocris, Milan, Italy), the inhibitor for the cyclin-dependent kinase Cdk5, Roscovitine (20 μM, Sigma-Aldrich), the two atypical antipsychotics RSP and CZP (respectively 20 nM and 5 μM, gifted by Dr. M. Leopoldo the University of Bari), and the selective inhibitor for ERK1/2, U0126 (30 min pretreatment with 10 μM). Control cells were treated with DMSO as the vehicle of the drugs. Vehicle and drugs, alone or in combinations, were added in pre-warmed fresh medium.

2.4. Immunofluorescence and morphological analysis

Postnatal and embryonic cells were rinsed twice in PBS and fixed in PFA 4 % for 20 min at RT. After permeabilization with 0.1 % Triton X-100 in PBS for 15 min, cells were incubated for 30 min at RT with a blocking solution of 3 % BSA in PBS. Primary antibodies in 1 % BSA-PBS solution were applied overnight at 4 °C. To label neurons, cells were incubated with anti-Beta tubulin III (anti-mouse Tuj1, 1:750, Sigma-Aldrich T8660). After three washes with PBS for 5 min, cells were incubated for 3 h at RT with fluorophore-conjugated secondary antibodies (Alexa Fluor 594, 1:400, ThermoFisher Scientific), diluted in 1 % BSA-PBS. Then, the cells were washed three times with PBS for 5 min and incubated for 10 min an RT with DAPI (4′, 6-diamino-2-fenilindolo, 1:1000), washed 5 times with PBS and mounted on glass slides with a mounting solution. As a negative control for the immunofluorescence, cells were incubated with the secondary antibody alone.

Fluorescent signals from Tuj1 stained neurons were detected with a fluorescence microscope (Leica DM6000B, Wetzlar, Germany) equipped with an objective 20x. Images were acquired with a high-resolution camera using the Leica Application Suite and software analyzed by Image J's image-processing software as previously described (Di Rita et al., 2020; Speranza et al., 2015).

Briefly, the neuronal cells were recognized by their immunoreactivity with Tuj1 antibody, and a total of 15 fields for each cell-culture condition were selected from at least three independently treated culture wells. The images were pre-processed using the Image J software to optimize illumination and contrast. The length of the neurites was estimated by measuring the length of a line manually drawn from the soma to the end of the primary neurites (neurites that originate directly from the soma) using the "Measure" function of the software. The analyses were carried out blindly to avoid any subjective influences during the measurements. Morphometric parameters were always compared to the controls from the same batch of dissociated cells treated with the vehicle alone for the same time length (CTRL).

2.5. RNA Isolation and RT-PCR analyses

RNA was extracted from primary cell cultures four days after seeding, using the Tri-Reagent according to the manufacturer's instructions (Sigma-Aldrich).

The analyses were always carried out in triplicate samples for each experimental point. Reverse transcriptase and quantitative real-time PCR analyses were performed as previously described (Speranza et al., 2013). Briefly, 2 μg of RNA was reverse transcribed using random hexanucleotides as primers (New England Biolabs Inc., 6 μM) and 200 U of Moloney-murine leukemia virus reverse transcriptase (New England Biolabs Inc.). Negative amplification control was performed in the absence of a template. Specific primer sets were designed with Oligo 6 software to obtain amplicon fragments with comparable size (around 100 bp). The primers used for the real-time PCR analyses are listed in Table 1.

SYBR green real-time PCR reactions were performed in 96-well plates using a 7900 Fast Real-Time PCR System (Applied Biosystem). Each cDNA sample was amplified in triplicate, and every reaction included a negative control (having no template) to eliminate the possibility of contamination. Thermal cycling conditions comprised the initial step at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.6. Statistical analyses

All the statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software). The differences among drug-treated and vehicle-treated cultures were assessed by One-Way ANOVA followed by Dunnet's post-hoc test and Two-Way ANOVA followed by Tukey's multiple comparison test. The significance threshold was set at $p < 0.05$.

3. Results

3.1. Dose-dependent effects of Risperidone and Clozapine on neurite outgrowth in embryonic cortical neuronal cultures

To investigate the effect of CZP and RSP on neuronal remodeling, we used primary cultures freshly isolated from the prefrontal cortex (CTX) of the E15 mouse brain. As a first step, we investigated in these cultures the presence of serotonergic receptor 7 (5-HT7R) as well as dopaminergic receptors. By real-time PCR, we detected the presence of 5-HT7R mRNA (Fig. 1) in agreement with previous data indicating 5-HT7R positive staining in embryonic cortical cultures (Speranza et al., 2013). The expression level of 5-HT7R mRNA was similar to that observed in the adult midbrain, used as a positive control (Fig. 1). Cortical neurons also showed a remarkable mRNA expression of D_1 and D_2 dopaminergic receptors, well-known molecular targets of RSP and CZP, while the dopaminergic receptor D_4 mRNA was faintly detectable (Fig. 1).

Table 1
Primers (5′-3′) used for real-time PCR.

Gene of interest	Forward 5′-3′	Reverse 5′-3′
5-HT7R	GCGGTCATGCCTTTCGTTAGT	GGCGATGAAGACGTTGCAG
DRD_1	CTGGCACAAGGCAAAACCTAC	TGTCATCCTCGGCATCTTCC
DRD_2	GCACAGCAAGCATCTTGAACC	CAACATAGGCATGGCCACAG
DRD_4	AAGGGAGCGCAAGGCAAT	AGAAAGGCGTCCAACACACC

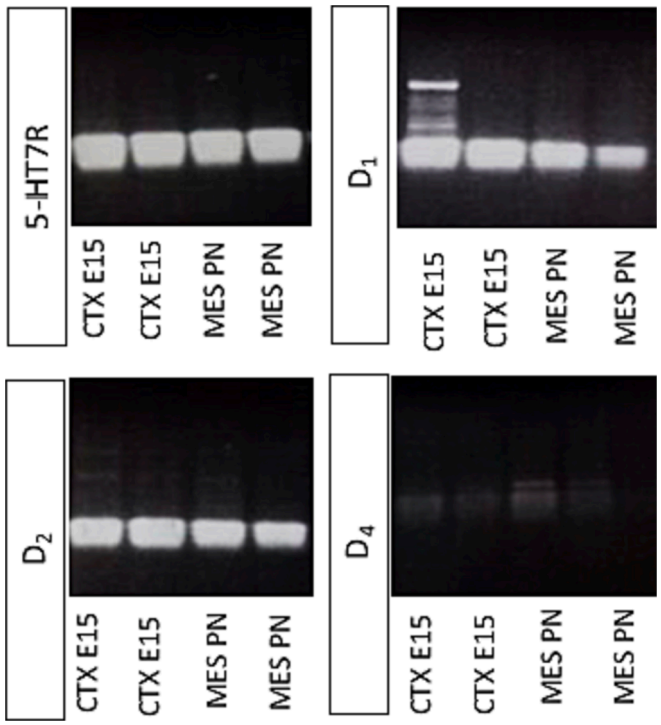

Fig. 1. mRNA characterization of embryonic neuronal primary cultures (E15) derived from cortex. Amplified fragments (71 bp) separated on a 2 % agarose gel. D_1: Dopamine receptor, subtype D_1; D_2: Dopamine receptor, subtype D_2; D_4: Dopamine receptor, subtype D4; 5-HT7R: Serotonin receptor, subtype 7; CTX E15: Embryonic cortex (E15); MES PN: Adult mesencephalon.

To evaluate the dose–response effect of RSP and CZP on neuronal morphology, E15 cortical primary cultures were incubated for 4 h with increasing concentrations of RSP (20 nM, 100 nM, and 500 nM) and CZP (500 nM, 1 μM, 5 μM, and 10 μM). The neurite elongation was analyzed using Tuj1 antibody staining. RSP, at the concentration of 20 nM, decreased the neurite length by about 20 % compared to controls (Fig. 2A, C). The decreasing effect remained unchanged when a higher drug concentration was utilized (Fig. 2A). Instead, 1 μM or higher concentrations of CZP increased the neurite length by about 30 %, compared to the control (Fig. 2B, C), while 500 nM of CZP did not affect the neurite length of the same neurons (Fig. 2B). In light of these findings, the subsequent experiments were conducted by incubating the cells for 4 h with RSP 20 nM, and CZP 5 μM.

3.2. *Effects of Risperidone and Clozapine in postnatal neuronal cultures*

Embryonic cultures often display high variability compared to postnatal cultures, due to developmental heterogeneity, variations in cell proliferation rates, higher sensitivity to environmental conditions, and the challenges associated with handling and isolating very small and fragile tissues. Thus, to reduce response variability, we performed our experiments using postnatal cultures, which are more homogenous, with cells that have started establishing mature synaptic connections. Moreover, to respect the principle of the 3R (Replace, Reduce, Refine) aiming to minimize the use of experimental animals and to explore the potential impact of RSP and CZP on neuronal morphology during early postnatal (P) development, we used primary cultures obtained from the prefrontal cortex of mouse pups aged between P0 and P3. We limited our experiments to P0-P3 pups to enhance in vitro culture quality. Furthermore, it has been reported that the cortical gene expression of 5-HTR7 does not change from P0 to P14 (Olusakin et al., 2020).

In P0-P3 postnatal cultures, we evaluated the CZP and RSP-induced changes in neurite length. As expected, the analysis of neurite length in these primary cultures confirmed the findings previously obtained in embryonic cultures. After 4 h treatment, RSP shortened the neurite length by approximately 20 %, while CZP increased the neurite length by about 20 % compared to the CTRL neurons (Fig. 3). It is interesting to note that the effect of CZP on neurite length was similar to that induced by the 5-HT7R selective agonist LP-211 (100 nM), previously described in embryonic and postnatal cultures (Speranza et al., 2013, 2015, 2017). Indeed, the treatment with LP-211 increased the neurite length by approximately 30 % in cortical neurons compared to the CTRL (Fig. 3), in line with our previous results (Speranza et al., 2013).

3.3. *Effects of Risperidone and Clozapine on neurite outgrowth: Contribution of 5-HT7R*

To test whether the effect of RSP (20 nM) and CZP (5 μM) on neurite outgrowth is 5-HT7R-dependent, we incubated the cortical neurons in the presence of SB-269970 (100 nM), a selective antagonist of 5-HT7R, alone or in combination with RSP or CLZ. In particular, after 3 days in vitro, the cultures were pre-treated with the SB-269970 or vehicle for 30 min and subsequently treated for 4 h with 20 nM RSP, 5 μM CLZ, or 100 nM LP-211.

The administration of the SB-269970 alone significantly decreased the neurite length compared to the CTRL by approximately 14 %, suggesting that 5-HT7R is constitutively activated in cortical postnatal cultures (Fig. 4A). The selective effect of SB-269970 on 5-HT7R was confirmed by experiments in which P0 cortical neurons were incubated for 4 h in the presence of LP-211 (100 nM), with or without SB-269970. Indeed, the increased neurite length LP-211-dependent was completely reversed by the concurrent incubation with SB-269970 (Fig. 4A), in line with our previous results (Speranza et al., 2013).

Then, the neurons were incubated with the antipsychotic drugs and the 5-HT7R antagonist SB-269970. Interestingly, the reduced neurite length detected in P0 cortical neurons incubated for 4 h in the presence of RSP did not change when neurons were concurrently incubated with SB-269970 (100 nM) (Fig. 4). Therefore, the neurite shortening RSP-induced effect appears to be 5-HT7R-independent.

On the other hand, the co-application of SB-269970 and CZP blocks the 25 % CZP-mediated neurite outgrowth of P0 cortical neurons (Fig. 4), indicating that the neurite outgrowth CZP-induced was 5-HT7R-dependent.

3.4. *Modulation of neurite length by CZP requires ERK1/2 phosphorylation.*

The ERK 1/2 signaling is a crucial convergence point for various pathways involved in neurite outgrowth (Buchser et al., 2010; Colucci-D'Amato et al., 2003; Speranza et al., 2013). To investigate whether inhibition of ERK could interfere with the morphogenic effects of RSP and CZP, we used the selective ERK1/2 inhibitor, U0126. After 3 days in vitro, the P3 cortical cultures were pre-treated for 30 min with U0126 or its vehicle. Subsequently, the cultures were stimulated for 4 h with 20 nM RSP, 5 μM CZP, and 100 nM LP-211.

As shown in Fig. 5A, treatment with U0126 alone determines a significant reduction of neurite length in cortical cultures, suggesting that ERK phosphorylation modulates neurite elongation also in the absence of 5HTR7 stimulation (Speranza et al., 2013). The stimulatory effect of LP-211 on neurite elongation was abolished by co-treatment with U0126 (Fig. 5A), confirming the involvement of the ERK signaling pathway in 5-HT7R-mediated elongation (Speranza et al., 2015). The RSP-mediated decrease in neurite length was unaffected by co-treatment with the ERK inhibitor. In contrast, the stimulatory effect of CZP on neurite growth was abolished by co-treatment with U0126, mimicking the effect observed with LP-211 (Fig. 5).

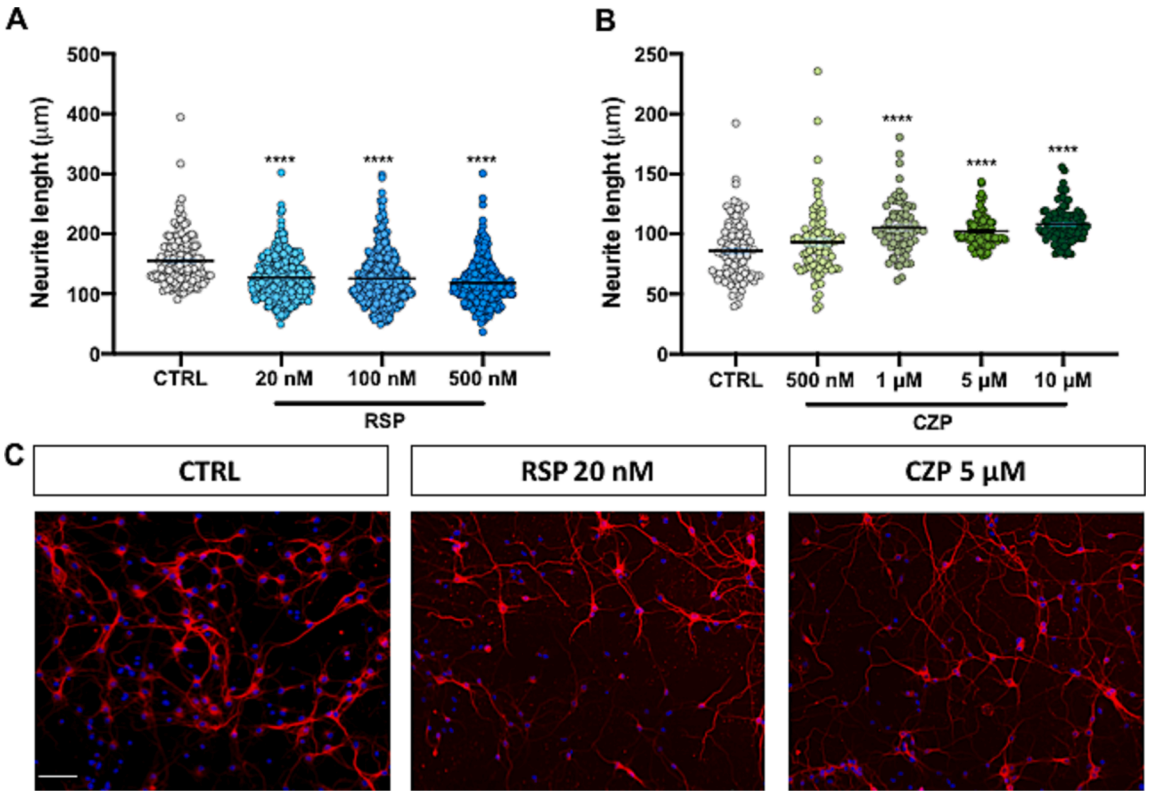

Fig. 2. Characterization and dose–response effect of Risperidone and Clozapine on neurite outgrowth of embryonic neuronal primary cultures (E15) derived from cortex. The cortical primary neuronal cultures from E15 mice, after 3 days in culture, were treated with various concentrations of Risperidone (A; RSP; 20 nM, 100 nM, and 500 nM) or Clozapine (B; CZP; 500 nM, 1 μM, 5 μM, and 10 μM) for 4 h. Neurite length was measured in cells stained with anti-Tuj1 antibody and expressed in micrometers (μm). Each experimental point was performed in at least three independent triplicates. The graphs represent means ± SEM from randomly selected fields for each cell culture condition (CTRL RSP n ≅ 180 neurites; RSP 20 nM n ≅ 300 neurites; RSP 100 nM n ≅ 300 neurites; RSP 500 nM n ≅ 300 neurites; CTRL CZP n ≅ 100 neurites; CZP 500 nM n ≅ 96 neurites; CZP 1 μM n ≅ 71 neurites; CZP 5 μM n ≅ 87 neurites; CZP 10 μM n ≅ 95 neurites). Asterisk (*) denotes values significantly different from the respective control (CTRL; vehicle only) determined by one-way ANOVA followed by Dunnett's post-hoc test (****$p < 0.0001$). (C) The panel shows representative images of cortical neurons treated with vehicle only (CTRL), RSP (20 nM), or CZP (5 μM) immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The images were acquired at a magnification of 20x. Scale bar: 50 μm.

3.5. Modulation of neurite length by CZP requires activation of Cdk5 kinase

The ERK signaling is linked to various signal transduction pathways, including the pathway involving cyclin-dependent kinase 5 (Cdk5), a serine/threonine protein kinase highly expressed in the brain. Cdk5 plays a critical role in regulating microtubule dynamics during ontogenesis (Drerup et al., 2010) and is involved in cytoskeletal reorganization (Cheung and Ip, 2007; Nikolic et al., 1996). In addition, Cdk5 activity is mandatory to promote the 5-HT7R-dependent effects on neuronal morphology (Speranza et al., 2017, 2015, 2013). To investigate whether Cdk5 inhibition could interfere with the effects of RSP and CZP on neurite outgrowth, we incubated postnatal P0 cortical primary cultures at 3 DIV for 4 h with roscovitine (Rosco, 20 μM), a selective inhibitor of Cdks. Rosco has affinity for two kinases of the same family, Cdk5 and Cdk2, but only Cdk5 is active in postmitotic differentiated neurons (Otyepka et al., 2006). Therefore, Rosco can be considered an exclusive inhibitor of Cdk5.

When cortical neurons were incubated with Rosco alone, there was a significant decrease in neurite elongation compared to the CTRL by approximately 33 %, indicating that Cdk5 activity is constitutively active (Fig. 6A).

In line with previous results (Speranza et al., 2013), it was observed that Rosco abolished the 5-HT7R-dependent neurite outgrowth induced by LP-211. Similar results were observed when primary cultures were co-treated with CZP and Rosco (Fig. 6). On the other hand, the reduced neurite length RSP-mediated was unaffected by co-incubation with Rosco (Fig. 6).

These findings suggest that the Cdk5 kinase is selectively involved in neurite outgrowth 5-HT7R and CZP-mediated, but not in reduced neurite length RSP-mediated.

4. Discussion

The morphology of neurons is responsible for the establishment of the correct neuronal connectivity. The complexity of dendritic branching, dendritic spines, and density of synaptic contacts influences the receiving input, the processing of information, and ultimately, cognitive functions of the nervous system, such as learning and memory. Indeed, various neurological disorders, including autism spectrum disorders, psychoses, attention deficits, mental retardation, and neurodegenerative diseases, exhibiting learning and memory alterations (Seabury and Cannon, 2020), often show significant alterations of dendritic branching patterns, including changes in dendritic length, arborization, and spine density (Cassoli et al., 2015).

This study investigated the effects of two atypical antipsychotic drugs, RSP and CZP, on neuronal cytoarchitecture. RSP and CZP are commonly used to treat schizophrenia and bipolar disorder. They act by modulating neurotransmitter levels, including dopamine and serotonin, in the brain. We utilized primary cultures of neurons from the cortical area of brain rodents to assess neurite length, a hallmark of neuronal plasticity during the early stages of nervous system development.

Our findings demonstrate that CZP in embryonic and postnatal cultures from the mouse brain cortex promotes significant neurite

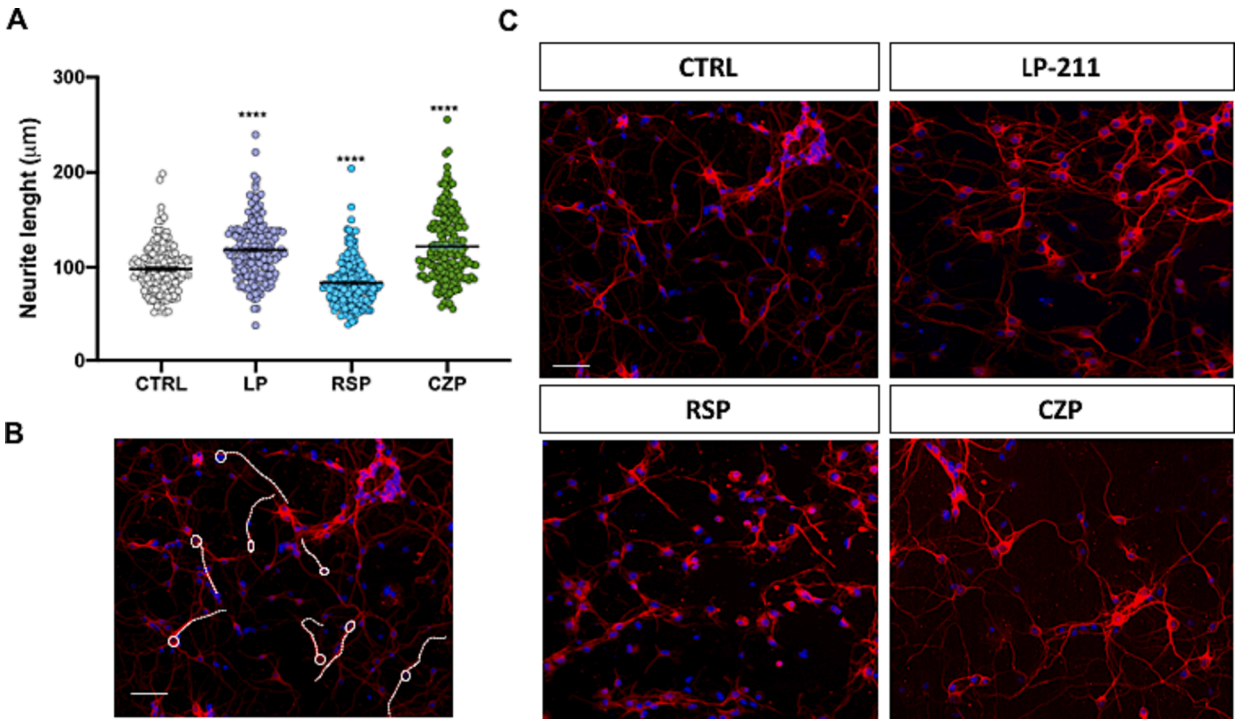

Fig. 3. Acute pharmacological stimulation with Risperidone and Clozapine in postnatal cultures. (A) Cortical primary neurons from P3 mice, after 3 days in vitro, were treated with 100 nM LP-211 (LP), 20 nM risperidone (RSP), and 5 μM clozapine (CZP) for 4 h. Neurite length was measured in cells stained with anti-Tuj1 antibody and expressed in micrometers (μm). Each experimental point was performed in at least three independent triplicates. The graph represents means ± SEM from randomly selected fields for each cell culture condition (CTRL n ≅ 150 neurites; LP n ≅ 180 neurites; RSP n ≅ 180 neurites; CZP n ≅ 180 neurites). Asterisk (*) denotes values that are significantly different from control (CTRL; vehicle only) by one-way ANOVA followed by Dunnett's post-hoc test (****$p < 0.0001$). (B) The panel shows a representative image of cortical neurons treated with vehicle only (CTRL) immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The dashed white lines are manually drawn by the operator from the soma (white circle) to the end of the primary neurite to measure neurite length. (C) The panels show representative images of cortical neurons treated with vehicle only (CTRL), LP-211 (100 nM), RSP (20 nM), and CZP (5 μM) immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The images were acquired at a magnification of 20x. Scale bar: 50 μm.

elongation, whereas RSP induces neurite retraction. The effects of CZP on dendritic spine formation in hippocampal neuron cultures have been previously described (Critchlow et al., 2006). Thus, our results provide novel insights into its effects on neuronal cytoarchitecture. Since both drugs interact with various serotonin receptors, including the 5-HT7R, which is well known to be involved in neuronal morphology remodeling (Crispino et al., 2020; Volpicelli et al., 2014; Wirth et al., 2017), we hypothesized that the antipsychotic-induced neuronal remodeling may be mediated by 5-HT7R. SB-269970, a selective antagonist of 5-HT7R, was used to verify this hypothesis. Our results indicate that the morphological effects of RSP were independent of 5-HT7R, while this receptor partially mediates the effects of CZP. These findings suggest that the signaling pathways responsible for neurite elongation and retraction are distinct, in agreement with previous results on growth cone elongation and retraction (Wirth et al., 2017).

Whole-cell radioligand binding studies have suggested that risperidone interacts in an irreversible or pseudo-irreversible manner with the 5-HT7R, producing a rapid and long-lasting inactivation of the receptor (Smith et al., 2006). This inactivation inhibits cAMP production (Knight et al., 2009). RSP acts on numerous biochemical pathways (Kashem et al., 2009). Proteomic data obtained from neural stem cells (NSCs) treated with RSP reveal its impact on various proteins involved in cytoskeletal remodeling, including Dihydropyrimidinase 2 (DRP-2), a protein implicated in axonal differentiation, guidance, neurite elongation, remodeling, and repair (Johnston-Wilson et al., 2000; Weitzdoerfer et al., 2001).

CZP is described as an inverse agonist or non-inactivating antagonist of the 5-HT7R (Andressen et al., 2015; Krobert and Levy, 2002). Surprisingly, CZP elicits opposite effects than RSP since it promotes neurite elongation. This response is mainly mediated by the activation of the 5-HT7R, as co-treatment with the selective antagonists SB-269970 abolishes the neurite outgrowth while not affecting RSP-mediated responses. The apparent discrepancy between the CZP antagonistic effect on 5-HT7R and its capacity to stimulate neurite elongation could be attributed to the fact that 5-HT7R shows approximately 40 % homology with the 5-HT1AR. Moreover, most 5-HT7R ligands, such as CZP, also exhibit affinity for 5-HT1AR (Leopoldo et al., 2011; Mitroshina et al., 2023; Modica et al., 2018). 5-HT1AR and 5-HT7R can heterodimerize, and this phenomenon leads to a reduction in 5-HT1AR-mediated activation of Gi protein without affecting 5-HT7R-mediated signaling. As a result, the interplay between these two receptors and their ligands may give rise to diverse and sometimes contrasting outcomes in neurite elongation and other cellular processes.

The idea that 5-HT7R antagonism improves deficits associated with schizophrenia or dementia in association with multitargeted drugs has been confirmed by several studies showing that antipsychotic drugs act on multiple monoaminergic GPCRs (Okubo et al., 2021; Stiedl et al., 2015). SB-269970 preincubation induces functional desensitization of 5-HT7R, probably changing the receptor density (Tokarski et al., 2012) and blocking the CZP binding. This would block the positive effect of heterodimerization on neurite outgrowth.

Furthermore, given the extremely complex polypharmacological profile of CZP, we cannot exclude that it may act at the same time on 5-HT7R and other receptors, such as D_1, and D_2 that are expressed in our cells (Iasevoli et al., 2023).

Proteomic studies conducted on rodents have shown significant effects of CZP on the expression of various molecules involved in cytoskeletal reorganization, particularly those directly implicated in

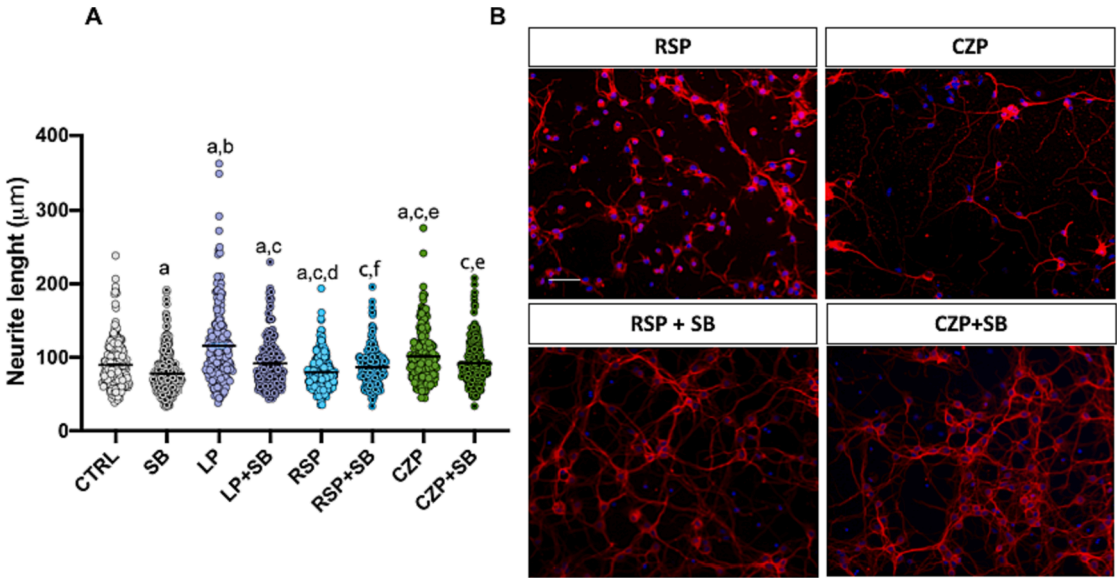

Fig. 4. Involvement of 5-HTR7 in Clozapine and Risperidone-mediated effects on neurite outgrowth. (A) Cortical cultures obtained from P0 mice, after 3 days in vitro, were treated with LP-211 (LP, 100 nM), Risperidone (RSP, 20 nM), and Clozapine (CZP, 5 μM), alone or in combination with the selective antagonist of 5-HT7R, SB-269970 (SB, 100 nM) for 4 h. The cells were pre-treated with SB for 30 min before the incubation with each drug. Neurite length was measured in cells stained with anti-Tuj1 antibody and expressed in micrometers (μm). Each experimental point was performed in at least three independent triplicates. The graph represents means ± SEM from randomly selected fields for each cell culture condition (n ≅ 250 neurites). The letter denotes values that are significantly different from control (CTRL, a), SB (b), LP (c), LP + SB (d), RSP (e), CZP (f) evaluated by two-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). (B) Representative images of cortical neurons treated with RSP (20 nM) and CZP (5 μM) alone and in combination with SB. Neurons were immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The images were acquired at a magnification of 20x. Scale bar: 50 μm.

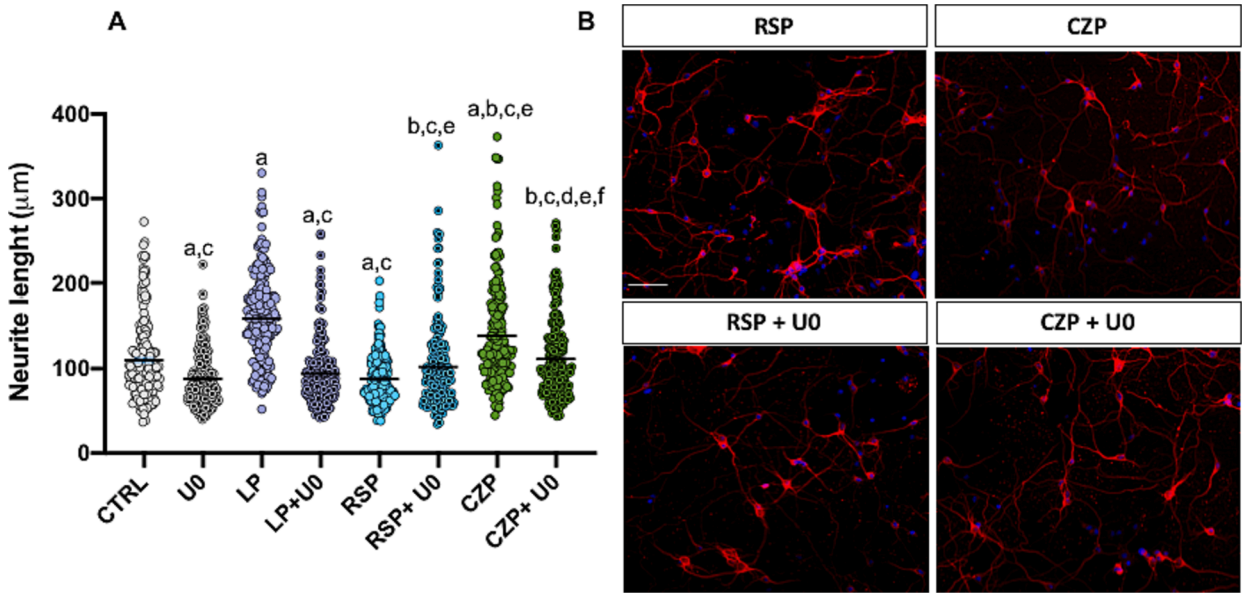

Fig. 5. ERK 1/2 signaling pathway is required for the modulation of neurite length induced by Clozapine. (A) Postnatal (P0) cortical cultures were treated after 3 days in vitro with LP-211 (LP, 100 nM), Risperidone (RSP, 20 nM), and Clozapine (CZP, 5 μM), alone or in combination with the selective ERK1/2 inhibitor, U0126 (U0). The cells were pre-treated with U0 for 30 min before the treatment. Neurite length was measured in cells stained with anti-Tuj1 antibody and expressed in micrometers (μm). Each experimental point was performed in at least three independent triplicates. The graph represents means ± SEM from randomly selected fields for each cell culture condition (n ≅ 200 cells). The letter denotes values that are significantly different from control (CTRL, a), U0 (b), LP (c), LP + U0 (d), RSP (e), CZP (f) evaluated by two-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). (B) Representative images of cortical neurons treated with RSP (20 nM) and CZP (5 μM) alone and in combination with U0126 (U0). Neurons were immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The images were acquired at a magnification of 20x. Scale bar: 50 μm.

synaptogenesis. For example, CZP regulates the phosphorylation of proteins mediating the collapsin response, stabilizing microtubules, and modulating extracellular protein signals, including semaphorins and neurotrophins. These proteins regulate the development of axons, dendrites, and spines by acting on cytoskeletal organization. CZP also increases the level of the alpha chain of spectrin (αII spectrin), a protein crucial for synapse development. Cell adhesion molecules and αII spectrin ensure proper localization of the presynaptic complex on the

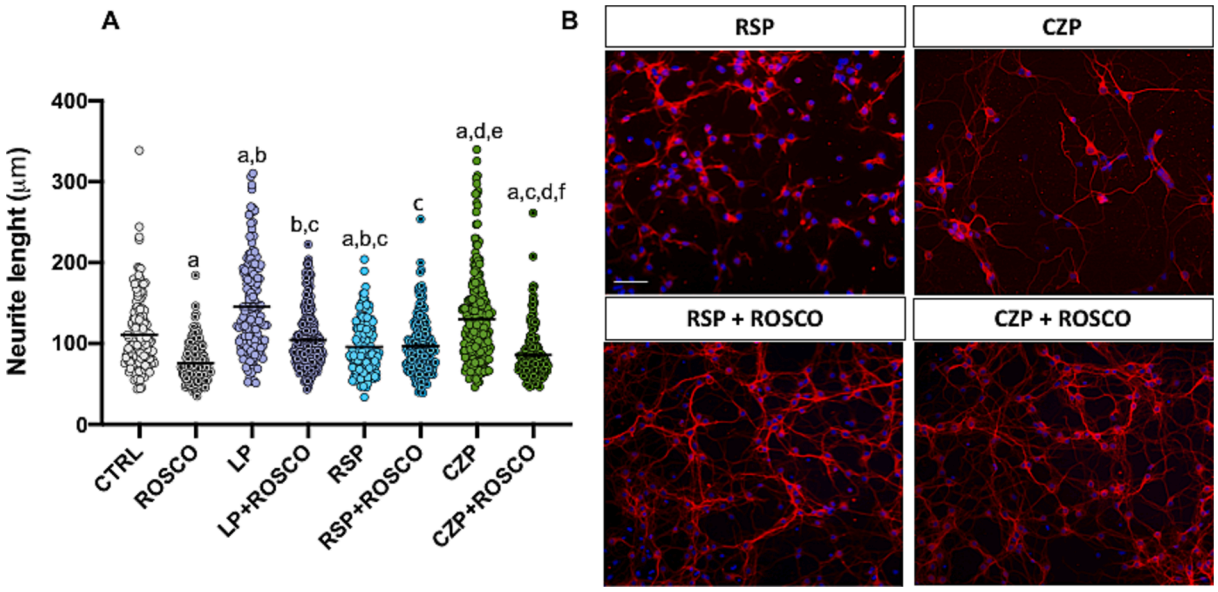

Fig. 6. Activation of cyclin-dependent kinase 5 (Cdk5) is required for the neurite remodeling mediated by Clozapine. (A) Postnatal (P0) cortical cultures were treated after 3 days in vitro with LP-211 (LP, 100 nM) Risperidone (RSP, 20 nM) and Clozapine (CZP, 5 μM), alone or in combination with the selective Cdk5 inhibitor, Roscovitine (Rosco; 20 μM). Neurite length was quantified in cells stained with anti-Tuj1 antibody and measured in micrometers (μm). Each data point represents the mean ± SEM from at least three independent triplicate experiments. The graph indicates randomly selected fields for each cell culture condition (n ≅ 185 neurites). The letter denotes values that are significantly different from control (CTRL, a), ROSCO (b), LP (c), LP + ROSCO (d), RSP (e), CZP (f) evaluated by two-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). (B) Representative images of neurons treated with RSP (20 nM) and CZP (5 μM) alone and in combination with Rosco. Neurons were immunostained with the neuronal marker Tuj1 (red) and counter-stained with the nuclear marker DAPI (blue). The images were acquired at a magnification of 20x. Scale bar: 50 μm.

neuronal membrane (Sytnyk et al., 2004). Altogether, these findings suggest that some of these molecules may mediate the observed stimulation of neurite outgrowth. In our experimental setting, it was shown that CZP promotes neuronal cytoskeletal remodeling by ERK phosphorylation with the involvement of Cdk5 kinase.

Further research will be addressed to fully understand the complex interplay between 5-HT1AR/5-HT7R heterodimerization and whether a modification in the receptor affinity can affect other morphological parameters, such as neurite branching and dendritic spines in cortical neurons. This knowledge will advance our understanding of the complexity of serotonin receptor signaling, the action of antipsychotic drugs, and their impact on neuronal connectivity. Altogether, this data may lead to improved treatments for a wide range of neurological and psychiatric disorders.

5. Conclusions

This study aimed to explore the impact of two antipsychotic drugs, Risperidone (RSP) and Clozapine (CZP), on neurite outgrowth in embryonic and early postnatal neuronal cultures derived from rodent brains, and the molecular mechanisms underlying their effects with the possible involvement of 5-HT7R. Our findings indicate that CZP can enhance neurite outgrowth, and this effect is, at least partially, mediated by 5-HT7R, and involves the ERK and Cdk5 pathways. Conversely, RSP induces neurite retraction in a 5-HT7R-independent manner. The differential impact of these two atypical antipsychotics on neuronal morphology suggests their distinct roles in synaptic plasticity, neuronal signaling, and circuitry formation, processes that are dysregulated in psychotic disorders characterized by disruptions of neuronal connectivity, such as schizophrenia. Furthermore, our results propose that the multiple or selective modulation of the 5-HT7R signaling pathways could offer a promising molecular strategy to ameliorate cognitive impairments in individuals affected by psychotic disorders.

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CRediT authorship contribution statement

Luisa Speranza: Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Marta Molinari:** Validation, Formal analysis, Data curation. **Floriana Volpicelli:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Enza Lacivita:** Writing – review & editing, Resources, Methodology. **Marcello Leopoldo:** Writing – review & editing, Resources, Methodology. **Salvatore Pulcrano:** Writing – review & editing. **Gian Carlo Bellenchi:** Review & editing. **Carla Perrone Capano:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Marianna Crispino:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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